



Supporting Online Material for

Engineering Cooperativity in Biomotor-Protein Assemblies

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Materials and Methods

All cloning procedures for the biopolymer templates as well as the monomeric kinesin motor constructs were carried out in the *E. coli* strain XL1-Blue (Stratagene). The resulting plasmids were transformed into competent BL21 cells which were used as the expression host strain. The scaffold proteins and the motors were expressed and purified separately from one another. Motors assemblies were formed in solution by incubating the motor and the scaffolds in solution prior to measurements. Microtubules were formed by polymerizing phosphocellulose-purified tubulin that was either purified from cow brain (1) or purchased from Cytoskeleton.

Construction of $(Z_R-ELF_5)_n$ Plasmids. Genes encoding the artificial protein templates were constructed from synthetic DNA oligomers. The sequences of the plasmids produced from all cloning steps were verified using restriction mapping and DNA sequence analysis.

Construction of the polymer genes began by modifying a pQE60 vector (Qiagen). Two *BanI* restriction sites that are outside of the cloning region were removed using PCR, and a single *BanI* restriction was inserted into the cloning region by ligation of a synthetic oligonucleotide between the *BamHI* and *BglII* restriction sites of pQE60. This vector was designated pMD1. These modifications allowed a 75-bp oligonucleotide that encodes a single ELF element, (VPGVG)₂VPGFG(VPGVG)₂, to be ligated into the newly introduced *BanI* restriction site. After sequence verification, the ELF cassette was isolated by *BanI* digestion, and then self-ligated to form multimerized ELF cassettes

containing 2 to 7 ELF repeats. The ELF₅ cassette was isolated from an agarose gel, and ligated back into pMD1. The genes encoding Z_R were constructed by assembling synthetic oligoneucleotides through PCR as previously reported (2). The 140-bp fragment encoding Z_R was isolated using *Bam*HI digestion and ligated into pMD1 at the 5' side of the ELF₅ gene. The resulting Z_R-ELF₅ gene was PCR amplified from this vector. This cloning step modified the 3' end of the Z_R-ELF₅ gene by introducing a codon encoding a cysteine and a stop codon. This PCR product was excised by *Eco*RI and *Hind*III digestion and then ligated between the *Eco*RI and *Hind*III sites of pQE32 (Qiagen).

In order to prepare genes encoding for the dimeric (Z_R-ELF₅)₂ and trimeric (Z_R-ELF₅)₃ polymers, three additional PCR reactions were performed. These reactions modified either the 5', the 3', or both ends of the monomeric Z_R-ELF₅ fragment by introducing *Nhe*I restriction sites. All PCR products were *Nhe*I digested, while the 5' and 3' modified Z_R-ELF₅ fragments were also *Sph*I and *Hind*III digested respectively. In a single ligation reaction, the corresponding fragments were ligated together to form either a 1077-bp fragment encoding (Z_R-ELF₅)₂, or a 1611-bp fragment encoding (Z_R-ELF₅)₃, and inserted between the *Sph*I and *Hind*III restriction sites of pQE70. This synthetic step introduces four amino acids(KASK) between Z_R-ELF₅ units. Amino acid sequences for the final constructs are given in Table S1.

ELF:	(VPGVG) ₂ VPGFG(VPGVG) ₂
Z_R:	LEIRAAALRRRNTALRTRVAELRQRVQRLRNEVSQYETRGPL
Z_E:	LEIEAAALEQENTALETEVAELEQEVQRLENIVSQYRTRFGPL
Z_R-ELF₅	MDPLK-(Z _R -ELF ₅)-GVGC
(Z_R-ELF₅)₂	MHPLK-(Z _R -ELF ₅)-KASK-(Z _R -ELF ₅)-GVGC
(Z_R-ELF₅)₃	MHPLK-(Z _R -ELF ₅)-[KASK-(Z _R -ELF ₅)] ₂ -GVGC

Table S1. Final DNA constructs used to synthesize polymeric scaffolds

Construction and Expression of the K350-Z_E plasmid: The minimal motor domain containing the first 350 *N*-terminal amino acids of the *Drosophila* kinesin was cloned out of pEY4 (3) using PCR amplification. The pEY4 plasmid was provided kindly by Jeff Gelles. The 5'-primer (5'- ACATGCATGCCCCGCGGAACGAGAGATTCCCCGCG-3') introduced a *SphI* site at the translational initiation site. The 3'-primer (5'- CGCGGATCCATAGCGTCGCTTCCATTCTCGGCAGTAAG-3') was complementary to amino acids 320-350. This primer introduces a *BamHI* site after amino acid 350 in the neck region of the motor. A proofreading polymerase (Pfu turbo, Stratagene) was used during amplification to reduce the frequency of mutation. After digestion with *SphI* and *BamHI*, the PCR product was ligated into a pQE70 expression vector. The Z_E cassette was inserted into the *BamHI* site at the 3' end of the motor sequence. The final K350-Z_E construct encodes the first 350 amino acids of the motor, 44 amino acids of Z_E and a C-terminal 6xHis tag.

Expression and purification of the K350-Z_E motor were carried out essentially as described by Case *et al.* (4) with minor modifications. Protein synthesis and purification

were confirmed by SDS-PAGE analysis. For some experiments, a round of microtubule affinity purification was performed. Motor protein concentrations were determined using the Bradford method.

Bacterial Expression and Purification of $(Z_R-ELF_5)_n$ Proteins. Preliminary bacterial expression and purification of Z_R-ELF_5 failed to produce the full length monomer constructs due to proteolytic degradation of the basic zipper. The use of denaturing purification conditions did not prevent proteolysis, indicating that proteolytic cleavage of the basic zipper occurs *in vivo*. To overcome this problem, the $(Z_R-ELF_5)_n$ genes were coexpressed with a gene that encodes $Z_E-6xHis$. The $Z_E-6xHis$ gene was obtained by PCR amplification, and includes the bacteriophage T5 promoter of the pQE plasmids. The PCR product was *HindIII* digested and ligated into pREP4 (Qiagen) at a *HindIII* site. Both plasmids were cotransformed into *E. coli* Strain BL21 and expressed simultaneously upon addition of 1 mM isopropyl- β -thiogalactoside (IPTG). Expressing these genes together allows formation of the Z_E / Z_R coiled-coil complex *in vivo*. As a complex these polypeptides are highly resistant to proteolysis, and full length constructs are obtained.

The two plasmid system described above was used for bacterial expression of all three polymer scaffolds. During a typical synthesis, cell cultures were grown at 37°C in 2 L of TB medium [24 g of casein hydrolysate, 12 g of yeast extract, 3 g/L glycerol, KH_2PO_4 K_2HPO_4], and antibiotics [200 mg/L ampicillin and 35 mg/L kanamycin]. Once the cultures reached optical densities (600nm, OD_{600}) greater than 1, protein expression was induced for a period of 8 hours. The cells were then pelleted (4,000g for 10 min), resuspended in lysis buffer [8 M urea, 0.1 M Na_2HPO_4 (pH 8.0)], and lysed overnight

with vigorous agitation. The cell lysate was clarified by centrifugation and applied to a column of nickelnitrilotriacetic acid (Ni^{2+} -NTA) resin. After, washing with approximately 3 column volumes of lysis buffer, 1 column volume of a buffer containing 6 M guanidine-HCl and 0.1 M Na_2HPO_4 (pH 8) was added, and allowed to incubate for 10 min. During this step, the zippers in the heterodimeric $\text{Z}_\text{E}/\text{Z}_\text{R}$ complex disassociate, allowing the Z_E -6xHis and $(\text{Z}_\text{R}\text{-ELF}_5)_\text{n}$ proteins to be separated. The majority of the Z_E -His oligopeptide remains on the column, while the eluted $(\text{Z}_\text{R}\text{-ELF}_5)_\text{n}$ solutions are collected. Solutions of monomeric $\text{Z}_\text{R}\text{-ELF}_5$ polymers were dialyzed against ddH₂O using an 8,000 MW cutoff membrane, while the larger molecular weights of the dimeric and trimeric polymers permitted the use of a larger, 14,000 MW, cutoff membrane. After dialyzing the solution for 3 days with extensive water changes, protein impurities that precipitated from solution were removed by centrifugation (30,000g). Proteins samples were frozen and lyophilized for storage at 4°C.

Polymer stock solutions were made by resuspending the lyophilized polymers in 8M urea at concentration of 10 mg/mL. After a second round of dialysis against ddH₂O, the solutions were clarified (30,000g), and frozen for storage at -20°C. Purity of the stock solutions was determined using 9% tris-tricine gel electrophoresis and MALDI-TOF spectroscopy. The amount of Z_E -6xHis peptide that was carried over during purification was estimated by comparing the intensities of the Z_E -6xHis and $(\text{Z}_\text{R}\text{-ELF}_5)_\text{n}$ bands that were separated on protein gels. The Z_E -His peptide typically comprised less than 15% of the total protein in a monomeric $\text{Z}_\text{R}\text{-ELF}_5$ polymer solutions. The signal from the Z_E -His peptide could not be distinguished from background for stock solutions of the dimeric $(\text{Z}_\text{R}\text{-ELF}_5)_2$ and trimeric $(\text{Z}_\text{R}\text{-ELF}_5)_3$ polymers, indicating these solutions contain only

trace amounts of Z_E-6xHis. Any Z_E-His protein that did elute with the sample using this protocol can be removed by resuspending the protein in 6 M guanidine-HCl and 0.1 M Na₂HPO₄ (pH 8) and performing a second round of Ni²⁺-NTA purification.

Polymers were labeled with biotin at their C-terminal cysteine using a PEO-biotinmaleimide (Pierce) and standard maleimide labeling protocols (5).

Measurement of ATPase Hydrolysis Rates. ATPase assays were performed using a coupling system of pyruvate kinase and lactic dehydrogenase as previously reported (6). Reactions were carried out in 20 mM Tris-Cl (pH 7.0), 1 mM EGTA, 4 mM MgCl, 1 mM dithioerithritol and 1 mM ATP at room temperature. The concentration of the motor in the final reaction mixtures ranged from 0.05 to 0.2 μM from assay to assay. The concentrations of (Z_R-ELF₅)_n polymers were selected to give a one to one stoichiometry between the Z_R sites of the polymer and the motor. The final reaction mixture, minus microtubules, was equilibrated to room temperature. Reactions were then initiated by addition of microtubules.

Reaction rates were adjusted to account for background hydrolysis of the microtubules and normalized for the motor concentration. All assays were repeated using motor stocks prepared from multiple kinesin syntheses. A second round of microtubule affinity purification was used to verify that ATPase measurements are not limited by the percentage of active motor protein in solution (Table 1).

Microtubule Gliding Velocities. Microtubule gliding was observed by monitoring the motions of rhodamine labelled microtubules (Cytoskeleton) on top of a motor/polymer coated coverslip using objective-based total internal reflectance

microscopy. The motor/polymer complexes were attached to the coverslip surface through a biotin streptavidin linkage. Streptavidin coated reaction chambers were prepared as previously described (3). Before introduction into the chambers, motor/polymer assemblies were prepared in solution by incubating a 5 to 20 fold excess of K350- Z_E with biotin-labelled polymer templates for 20 min at 4°C. After incubation, these solutions were perfused into the flow cells and then incubated for 20 min at 4°C. A control experiment was performed using an unsaturated dimeric polymer. Here, a motor/polymer solution was prepared using the dimeric polymer (Z_R -ELF₅)₂, where the solution contained a three-fold excess of the Z_R attachment sites. In this case, the polymer/motor solutions were perfused into reaction chambers immediately after mixing the stock solutions, allowing attachment to the cover slip to compete with motor-polymer binding. Multiple motor stock solutions, obtained from several kinesin syntheses and purifications were used in these assays. Microtubule binding was not observed when either the polymers or neutravidin was omitted from the sample preparation.

After binding the motor/polymer complexes to the glass surface, the chambers were rinsed with 1 mg/mL casein, and then briefly incubated with taxol stabilized microtubules. Microtubule gliding was initiated by flushing with imaging buffer (80 mM Pipes, 1 mM EGTA, 4 mM MgCl₂, 5 mM Mg-ATP 10 µM Taxol and an oxygen scavenger system). Filament positions were recorded at 3 to 12 frames/sec using an Optronics camera outfitted with an intensifier. Microtubule positions and velocities were measured using RETRAC software (available online at <http://mc11.mcri.ac.uk/motorhome.html>), or a using custom routine in ImageJ (available online at <http://rsb.info.nih.gov/ij/>).

Temperature dependent assays were performed by controlling the temperature of the sample from both the top and bottom sides of the perfusion chamber. This was accomplished by adjusting the temperature of the oil immersion objective with a metal heating/cooling block. A second block contacted the back side of the sample. Both heating/cooling elements were connected in series to a temperature controlled circulating water bath. The temperature was measured at the objective using a silicon diode connected to a Lake Shore 340 temperature control. Several K-type thermocouples were placed around the sample and heating / cooling blocks to detect any thermal gradients. The temperature of the apparatus was stable to less than 50 mK over the full range of temperatures used.

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